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13. ABSTRACT *(Maximum 200 words)*

Since tumor cell migration and growth are dependent upon the formation of new blood vessels, it should be possible to manipulate invasive breast carcinoma by the inhibition of angiogenesis. This premise assumes that the restriction of the nutrients derived from blood should sustain the regression of solid tumors in man. Indeed, studies with human breast carcinoma have demonstrated an excellent correlation between tumor size, metastasis and support from the capillary network which comprise the microvasculature. It has also been established that tumor size and metastasis can be regulated by the migration, growth, and differentiation of the endothelial cell, effectors that either repress endothelial cell migration and/or growth or modify endothelial cell differentiation promise to provide new therapies for the clinical management of human angiogenic-dependent diseases. We have recently identified a novel hormone-receptor pathway which is involved in the regulation of angiogenesis. This hormonal signaling pathway is evolutionarily conserved between human and invertebrates and is a major pathway that signals the fate of cells during development. We have also identified the genes that may enable this pathway to be utilized specifically by the endothelial cell and interestingly, these genes are utilized by the mouse mammary tumor virus to manipulate similar events during the viral induction of mammary carcinoma in mice. Indeed, preliminary data suggest that the manipulation of one of these genes in cell culture regulates the migratory phase of the angiogenesis pathway. Thus, support is requested to confirm the hypothesis that Jagged-induced signaling through the endothelial cell-specific Notch-4 receptor antagonizes the chemotactic activity of the proangiogenic fibroblast growth factors. We suggest that these results may ultimately provide new and novel approaches to the clinical management of human breast carcinoma by the regulation of tumor angiogenesis.

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David A. Mears 7/1/99

06-11-99

PI - Signature Date

Table of Contents

Introduction:

The object of this proposal is to assess the role of Jagged-1 as a modifier of angiogenesis, an event important for the development of breast tumor growth and metastasis.

Body:

The following have been accomplished:

(i) construction of stable ECV and NIH 3T3 soluble Jagged-1 transfectants

(ii) construction of stable int-3/Notch transfectants

(iii) expression of multiple myc-epitope-tagged soluble Jagged-1 protein

Key Research Acoomplishments:

(i) soluble Jagged-1 induces collagen type 1-dependent tube formation in NIH 3T3 and ECV cells in vitro

(ii) soluble Jagged-1 induces a novel angiogenic pattern of neovessels in the chick CAM assay

(iii) soluble Jagged-1 induces a FGF-1-dependent transformation of NIH 3T3 cells in vitro

(iv) soluble Jagged-1 represses the migration of serum-induced NIH 3T3 cells

Reportable Outcomes:

(i) Wong, M.K.K., Prudovsky, I., Vary, C., Booth, C., Liaw, L., Mousa, S., Small, D., and Maciag, T. (1999) J. Biol. Chem.(Rapid Commun.) submitted.

Conclusions:

(i) Jagged-1 is a potent angiogenesis factor

(ii) Jagged-1 modifies the endothelial cell differentiation pathway

(iii) Jagged-1 functions as a non-transmembrane protein as an effector of Notch receptor signaling

References:

See Appendix

Appendix:

Enclosed are the appropriate copies of Wong, et al.

To: *J. Biol. Chem., Rapid Commun. (1999)*

Jagged-1 Regulates the Formation of Matrix-Dependent Chord-Like Structures In Vitro and Angiogenesis *In Vivo*

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Abbreviations: chorioallantoic membrane, CAM; fetal bovine serum, FBS; fibroblast growth factor, FGF; open-reading frame, ORF; phosphate-buffered saline, PBS; polymerase chain reaction, PCR; serial analysis of gene expression, SAGE; vascular endothelial growth factor, VEGF.

ABSTRACT

Jagged-Notch interactions regulate a transmembrane ligand-receptor signaling pathway involved in the regulation of cell fate determination as well as myoblast and endothelial cell differentiation. To further examine the role of the transmembrane ligand, Jagged-1, in the regulation of endothelial cell differentiation (Zimrin, A.B., Pepper, M.S., McMahon, G.A., Nguyen, F., Montesano, R. and Maciag, T. (1996) J. Biol. Chem. 271:32499-32505), we stably transfected NIH 3T3 cells with a truncated form of Jagged-1, which results in the secretion of a soluble form of the protein. Comparison of gene expression by serial analysis demonstrated that pro-α-2(I) collagen was repressed in soluble Jagged-1 transfectants. When plated on extracellular matrices, soluble Jagged-1 transfectants formed prominent chord-like structures on type 1 collagen but not on fibrin, fibronectin or vitronectin. While the soluble Jagged-1 transfectants exhibited growth kinetics similar to control cells and were unable to grow in soft agar, the cells were less sensitive to contact inhibition of growth in vitro and soluble Jagged-1 allografts formed tissue masses in nude mice after a prolonged latency period. Because these tumor-like structures exhibited an abundance of host-derived microvascular endothelial cells, the angiogenic potential of the soluble Jagged-1 transfectants was assessed by implantation of the transfectants in the chick chorioallantoic membrane assay. Indeed, these cells were not only able to induce angiogenesis but were also able to direct the formation of large macrovessel-like structures. These data suggest that Jagged-1 is able to initiate angiogenesis by the organization of matrix-sensitive cell-cell interactions including its ability to promote the development of chord-like structures.

INTRODUCTION

Angiogenesis is an integral part of physiologic and pathologic processes such as embryonic development, wound repair, solid tumor growth, and chronic inflammation, and involves the ability of the endothelial cell to coordinate migration, proliferation, and differentiation pathways to form new vascular structures (1,2). While the ability of the angiogenic growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) to initiate endothelial cell migration and growth are well described (3,4), the identification of factors involved in the regulation of the tubular, chord-like vascular phenotype has been difficult to access. We have previously reported that the transmembrane protein, Jagged-1, a ligand for its transmembrane receptor Notch (5), is involved in the regulation of human endothelial cell differentiation in vitro (1). Jagged:Notch interactions mediate an evolutionarily conserved intercellular signaling pathway responsible for the regulation of developmental cell fate decisions in vivo (6) and cellular differentiation in vitro (7,8). During the cloning of the human Jagged-1 gene, two cDNA clones were isolated which contained identical deletions resulting in the insertion of 15 novel amino acids followed by a premature termination of the Jagged-1 sequence prior to the domain encoding the transmembrane and intracellular sequences (1). Since this truncated Jagged-1 cDNA contained the Jagged-1 signal peptide sequence, we anticipated that cells transfected with this construct would secrete the truncated ectodomain of Jagged-1 as a soluble and extracellular form of the Jagged-1 protein and this would eliminate the transmembrane constraints imposed upon the Jagged-1 ligand to signal by an intercellular pathway. We report that human soluble Jagged-1 is an angiogenesis factor in vivo which is able to influence the formation of a chord-like phenotype in vitro.

MATERIALS AND METHODS

Soluble Jagged-1 Plasmid Construction: The soluble myc epitope-tagged Jagged expression vector was generated using two separate sequential polymerase chain reaction (PCR) protocols. Overhang PCR was used to place a consensus Kozak sequence 5' to the Jagged-1 open-reading frame (ORF), and to truncate Jagged-1 immediately 5' to the transmembrane domain. This construct was assembled by ligating the PCR-modified 5' and 3' amplicon into the shuttle plasmid, MW27, which consists of the full-length Jagged-1 cDNA in pBlueScript and was subcloned into the eukaryotic expression vector pMexneo (9) using the newly engineered 5' EcoR1 and 3' Xho 1 sites to produce the final product. The forward primer used for the 5' modifications was 5'-GACTATGCGAATTCGGATCCGTCGACGCCACCATGGGTTCCCCA CGGACACGCG and reverse primer was 5'-CAAGTTCCCCCGTTGAGACA, where the Kozak sequence is underlined. The forward primer used for the 3' modification was 5'-ATGGACAAACACCAGCAGAA and reverse primer was 5'-TAGTGCTCGAGCTATTACAA GTCTTCTTCAGAAATAAGCTTTTGTTCATCTGTTCTGTTCTTCAG, where the myc epitope is underlined. The template used for PCR was the complete human Jagged-1 ORF originally obtained from Dr. G. Gray (Yale University). Reactions were performed using Vent polymerase (New England Biolabs) in 1X Vent buffer as recommended by the manufacturer. The thermal cycling parameters consisted of 94°C (1 min) followed by 35 cycles at 94 °C (30 sec), 62 °C (30 sec), 72 °C (30 sec) followed by a 10 min hold at 72 °C before termination at 4 °C. The 5' PCR-modified product was digested with EcoRI and BglII, electrophoretically resolved on a 1% (w/v) agarose gel, electroeluted, and ligated with a similarly digested MW27 to create MW13 using standard protocols (10). The modified 3' PCR-amplified product was similarly processed except that restriction digestion utilized XhoI and AccI. The ligation was

performed with a similarly digested MW13 to yield MW32. This 5'-Kozak-truncated Jagged-1 3'-myc-tagged pBlueScript construct was digested with EcoRI and XhoI and ligated into pMexneo. All restriction enzymes and buffers were obtained from New England Biolabs and two soluble Jagged-1 transfectant clones, 38-1 and 38-4, and one insert-less vector transfectant clone were used for experimentation.

Cell Transfection, Immunoprecipitation and Matrix Preparation: NIH 3T3 cells were transfected with MW38 using the calcium-phosphate kit (Stratagene) and G418 (Gibco/BRL) selection. Stable soluble Jagged-1 transfectants were grown and maintained in DMEM (GIBCO/BRL) supplemented with 400 μg/ml G418 and 10% (v/v) fetal bovine serum (FBS) (HyClone, Logan, UT). G418-resistant cells were grown to confluency in DMEM containing 10% (v/v) FBS, the cells washed twice in phosphate-buffered saline (PBS) and incubated with labeling media consisting of cys- and met-free DMEM supplemented with 1X Nutriderma (Gibco/BRL) and 0.4 μCurie/ml of [^{35}S]-met/cys mixture (DuPont-New England Nuclear). After 4 h, the labeling medium was removed, the cells washed once with ice cold PBS, scraped into 1.0 ml of PBS and pelleted. The cell pellets were resuspended in RIPA lysis buffer containing 1 mM PMSF, 10 μg/ml aprotinin and 1 μg/ml leupeptin (Sigma), clarified by centrifugation (13,000 xg for 10 min) and incubated with 30 ul of Protein-A Sepharose (Pharmacia) which had been complexed with 9E10 monoclonal antibodies to the Myc epitope (Oncogene). The immunoprecipitates were washed four times with RIPA buffer, dissolved in 50 μl of 2X SDS sample buffer, and resolved in 8% (v/v) SDS-PAGE as described (11).

To assess the secretion of soluble Jagged-1, the conditioned medium (1 ml) from the [^{35}S]-met/cys-labeled cells were collected in 1 mM PMSF and 10 μg/ml aprotinin then incubated

with 50 µl of Protein A-Sepharose and treated as outlined above except that the immunoprecipitates were washed six times prior to being dissolved in SDS sample buffer.

Cell culture dishes were coated with 10 µg/cm^2 of human fibronectin for 2 h, the fibronectin removed and the plates washed three times with sterile PBS. Collagen gels were formed in 6 well plates by mixing type I collagen (Vitrogen 100, Collagen Corporation), 10X DMEM (Gibco/BRL), and sodium bicarbonate (11.8 mg/ml) in a 8:1:1 (v/v) ration on ice and then quickly dispensed (1.5 ml) into the individual cell culture dishes. The collagen mixture was allowed to gel for 1 h prior to use. Soft agar growth assays were performed as previously described (12).

Serial Analysis of Gene Expression (SAGE): The SAGE method was performed as previously described (13,14). Briefly, polyA$^+$ RNA derived from insert-less vector control and soluble Jagged-1 NIH 3T3 cell transfectant-derived polyA+ RNA were converted to ds-cDNA (cDNA Synthesis System, BRL) using 5′-biotinyl-dT$_{18}$ (Integrated DNA Technologies, Inc.). The cDNA was cleaved with NlaIII, the 3'-biotinylated fragments captured on streptavidin-coated magnetic beads (Dynal), the bound cDNA was divided into two pools, and one of the following linkers containing recognition sites for BsmFI and a NlaIII complementary terminus was ligated to each pool: linker 1,5′-TTTGGATTTGCTGGTGCAGTACAACTAGGCTTAATAGGGACATG–3′, 5′ TCCCTATTAAGCCTAGTTGT ACTGCACCAGCAAATCC (amino-C7)-3′ and linker 2, 5′ -TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACATG-3′,5′TCCCCGTACATC GTTAGAAGCTTGAATTCGA GCAG (amino-C7)-3′. SAGE tags were released with BsmFI, the tag overhangs filled in with T7 polymerase, ligated using T4 DNA ligase (BRL) overnight at 25 °C, diluted and amplified by PCR for 28 cycles (primers: 5′-GGATTTGCTGGTGCA GTACAACT-3′ and 5′-CTGCTCGAATTCAAGCTTCTAAC-3′). The product was fractionated

by PAGE, the 104 bp, product containing two tags ligated tail to tail (ditag), excised and extracted from the gel, cleaved with NlaIII, and the ditags purified by gel electrophoresis, excised and self-ligated to produce ditag concatamers (13,14). The concatenated products were separated by PAGE, products between 300 bp and 800 bp were excised and cloned into the SphI site of pZero (Invitrogen). Colonies were screened for insert size by PCR with M13 forward and M13 reverse primers. Clones were introduced into 25 μl PCR reactions containing 0.5uM M13 forward and reverse primers and subjected to thermal cycling (25 cycles) consisting of 20 sec at 95°C, 1 min at 52°C and 1 min. at 72°C. Clones selected on the basis of insert size were subjected to automated fluorescent DNA sequence analysis using rhodamine dideoxynucleotide terminator chemistry according to the instruction of the manufacturer (Applied Biosystems, Inc.)

Sequence files were analyzed by means of the SAGE program group, which identifies the anchoring enzyme site with the proper spacing, extracts the two intervening tags, and records them in a database. The potential identities of the tags were established by their presence in GenBank or DbEST databases (release 109).

Assessment of Soluble Jagged-1 NIH 3T3 Cell Transfectant Behavior In Vivo: The soluble Jagged-1 NIH 3T3 cell transfectants were grown to confluence under G418 selection and 24 h prior to injection the medium was changed to DMEM containing 10% (v/v) FBS. The transfectants were washed with PBS, harvested by trypsin digestion and resuspended in sterile/pyrogen-free PBS prior to injection. The cells were >95% viable by trypan blue exclusion and were free of mycoplasma and indigenous murine viruses including mouse hepatitis, adenovirus, pneumonia, cytomegatovirus and sendai (Anmed/Biosafe Inc, Rockville, MD). Female athymic nude mice (nu/nu) between 8-12 weeks of age (NCI-FCRDC) received 150 mg/kg of cyclophosphamide in pyrogen-free water by the intraperitoneal route 24 h prior to

injection and 200 µl of cell suspension (10^6 cells) was injected intradermally into the right flank. Following euthanasia, tissue growths were exposed by dissecting along the subcutaneous tissue plane and the tissue masses removed, fixed in 10% (v/v) buffered formalin and processed for paraffin sectioning and hematoxylin and eosin staining. Representative portions of these masses were also embedded in O.C.T. compound (Miles Scientific, Elkhart, IN) and snap frozen in 2-methylbutane (E.M. Science, Gibbstown, NJ) on dry ice. Frozen sections were placed onto glass slides, fixed in chilled acetone and dried. Immunohistochemistry was performed using the ABC system (Vector) and a 1:200 dilution of a rat-derived anti-CD31/PECAM (Pharmingen).

The chick chorioallantoic membrane (CAM) angiogenesis assay was performed as previously described (15,16) and utilized $2.5x10^6$ lethally irradiated soluble Jagged-1 NIH 3T3 cell transfectants per CAM. Recombinant human FGF-2 (10 ng) and insert-less vector NIH 3T3 cell transfectants served as positive and negative controls, respectively. The assay was harvested 4 d post-implantation and the angiogenic index quantitated by computer-assisted morphometric analysis of vessel number.

RESULTS AND DISCUSSION

The soluble Jagged-1 transfectants were analyzed for Jagged-1 expression by immunoprecipitation of [^{35}S]-cys/met-labeled cells. As shown in Figure 1, SDS-PAGE analysis of the myc epitope immunoprecipitants resolved a band of approximately 130 kDa in both cell lysate and conditioned medium which corresponds to the size predicted by the mass of the soluble Jagged-1 myc epitope translation product. Analysis of differential gene expression by SAGE also revealed that the soluble Jagged-1 transfectants were able to differentially express 227 transcripts containing either known or novel sequences. These results have been posted (http:\\Zappa.mmcri.mmc.org\~varyc\sjag) and a selected number are listed in Table 1.

Interestingly, enhancer of split groucho (X73360), type IV collagenase (X84324), connexin 32 (M63802), cathepsin D (Z53337), and vimentin (X51438) were among the 163 known transcripts expressed with an enhanced level by the soluble Jagged-1 transfectants and pro-α-2(I) collagen (X58251), FGFR-1 (M33760) and IkB-β (U19799) were among the 64 known transcripts with apparent reduced levels of expression.

Since pro-α-2 (I) collagen expression appeared to be prominent among the repressed transcripts and collagen matrixes are known modifiers of cellular phenotype in vitro (17), we plated the soluble Jagged-1 transfectants on type 1 collagen. As shown in Figure 2, the soluble Jagged-1 transfectants exhibited a chord-like phenotype with the formation of an interlacing arborizing pattern. This chord-like phenotype was also observed when the soluble Jagged-1 transfectants were plated on plastic at low cell seed density (Figure 2) in which groups of cells organize into chord-like arrays one to two cells in width. While these structures progress through the arboring phase, the monolayer assumes a normal NIH 3T3 cell phenotype as the population density nears confluence. On occasion, these structures are readily visible in the confluent monolayer and can extend several millimeters in length. In contrast, soluble Jagged-1 transfectants did not exhibit a chord-like phenotype on either fibrin-, fibronectin- or vitronectin-coated surfaces (data not shown). Likewise, neither wild type NIH 3T3 cells (Figure 1) nor insert-less vector NIH 3T3 cells transfectants exhibit this chord-like phenotype either on plastic or on a collagen type-I matrix.

A comparative assessment of the proliferative potential of the soluble Jagged-1 transfectants with insert-less vector transfectants revealed that the population doubling time was not altered when cells were subconfluent and this was consistent with the absence of a transformed in vitro phenotype including the failure of the Jagged-1 transfectants to grow in soft

agar (data not shown). However, the soluble Jagged-1 transfectants were not sensitive to contact inhibition of growth (Figure 3) and as a result, we assessed their potential to form tumors in athymic nude mice. We observed that the soluble Jagged-1 transfectants were able to form tissue masses (Figure 4A) but only after an extended latency period of approximately 8 weeks. Full necropsy of these animals did not reveal any evidence of local or distant metastases and gross dissection of these tissue masses revealed prominent angiogenesis characterized by 1-2 large feeder vessels, each giving rise to a rich percolating network of smaller vessels visible on the surface of the tissue mass (Figure 4A). Histologic examination further revealed large numbers of capillaries on the surface that penetrated into the body of the tissue mass yielding slit-like blood spaces (Figure 4B). Immunohistochemical analysis of the endothelial cell-specific marker, CD31 (PECAM), revealed not only the presence of microvessels but also a plethora of CD31-positive cells organized as a collection of either noncontiguous single cells or sharply angulated short linear arrays (Figure 4C and D). Interestingly, unlike the well-formed intratissue mass microvessels, very few of these groups of CD31-positive cells contained blood, nor were they associated with intratissue mass blood spaces (Figure 4C and D).

Since primary in vitro cell isolates of the soluble Jagged-1 transfectants obtained from these tissue masses by G418 selection demonstrated their ability to form chord-like structures and re-implantation into nude mice demonstrated their ability to develop angiogenic tissue masses with a similar latency period (data not shown), we examined the angiogenic potential of the soluble Jagged-1 transfectants in the conventional chorioallantoic membrane (CAM) angiogenesis assay (15,16). As shown in Figure 5, the implantation of the soluble Jagged-1 transfectants yielded a prominent angiogenic response similar to the positive control, FGF-2 while the insert-less vector transfectants did not. Interestingly, the soluble Jagged-1 CAM also

exhibited the formation of prominent macrovessels, a novel and unusual feature which has not been previously observed with other angiogenic factors such as FGF (Figure 5) and VEGF(18).

Although we identified the human Jagged-1 transcript as a gene modified during the early stage of in vitro angiogenesis (19), the data reported here establish a role for the soluble form of Jagged-1 as an inducer of angiogenesis in vivo. Indeed, the function of Jagged-1 may be involved in directing the formation of a chord-like phenotype during the organization component of the non-terminal endothelial cell differentiation pathway (20). While transmission electron microscopic analysis of the chord-like structures revealed prominent interdigitations between cells with close membrane-membrane apposition (data not shown), a distinct lumen with interdigitation of the plasma membrane was not readily observed despite their resemblance to the tubular phenotype observed with in vitro populations of endothelial cells (21). We suggest that absence of a readily visible lumen in the chords formed by the soluble Jagged-1 NIH 3T3 cell transfectants may be either a consequence of another gene product, the absence of appropriate rheologic conditions or the absence of another genomic requisite not present in the NIH 3T3 cell. We further suggest that it is likely that another gene product may be responsible for lumen formation since the majority of the CD31-positive chord-like structures established in the soluble Jagged-1 tissue masses in vivo, also do not exhibit evidence of blood flow.

These data are consistent with the recent genetic observation that the Jagged-1 null mouse exhibits normal vasculogenesis but an abnormal and early lethal embryonic angiogenic phenotype including defects in the remodeling of the yolk sac and embryonic vasculature (20). Likewise, the observation that mutations in the human Notch-4 gene are responsible for the formation of CADASIL, a systemic vascular disease (22) is also consistent with the concept that Notch signaling is an important component of vascular physiology in man. It is also noteworthy

that the observation relating the repression of Jagged-1 function in human endothelial cells to an exaggeration of the ability of FGF but not VEGF to induce sprout formation also correlates well with the role of VEGF but not FGF as a mediator of vasculogenesis since the Jagged-1 null mice exhibit hemorrhage as a result of the failure to form the large vitelline blood vessels, a process mediated by angiogenesis (20). We suggest that this defect may ultimately involve enhanced endothelial cell sprout formation and a failure of the mutant vasculature to form chords.

The function of the ectodomain of Jagged-1 as a biological response modifier is also consistent with the recent observation (23) that the enzymatic function of kuzbanian, an ADAM metalloprotease gene family member (24), is required for the activity of the Drosophila Notch ligand, Delta. Although it is not known whether a proteolytic modification of the Drosophila Jagged-1 homolog, Serrate (25), requires a similar proteolytic modification, our data do suggest that the ectodomain of Jagged-1 may function in the absence of its transmembrane domain as an extracellular protein.

These data also add to the emerging concept that Notch-Jagged signaling may play an important role in neoplasia. Notch receptor expression is up-regulated in cervical cancer, Notch mutants can induce neoplastic transformation in the mammary and salivary glands, and Notch translocation is associated with human T cell lymphoblastic neoplasms (26). Recent studies with human cervical carcinoma specimens demonstrate that Jagged-1 is absent in normal cervix, and is overexpressed, along with Notch, in malignant cervical adenocarcinoma (27). The observation that the Jagged-1 transcript is present in metaplastic lesions argues that it may be involved in early pre-malignant lesion development. We therefore suggest that Jagged-1 may possess a multifaceted role in carcinogenesis by directly influencing cell-fate decisions in the neoplastic cells and by regulating endothelial cell chord development during angiogenesis.

FIGURE LEGENDS

Figure 1. Autoradiographs of myc-immunoprecipitates from NIH 3T3 cells grown in the presence of [^{35}S]-labeled cysteine and methionine. Stable myc-tagged soluble Jagged-1 (sol-jag) NIH 3T3 transfectants were compared to the parental wild-type NIH 3T3 cell line (wt). Conditioned media and cell lysates were obtained from the same population of cells. Arrow indicates the position of the [^{35}S]-cys/met-labeled soluble Jagged-1 protein.

Figure 2. Formation of Chords by Soluble Jagged-1 Transfected Cells. Vector-transfected control NIH 3T3 cells (A,C) and soluble Jagged-1 NIH 3T3 cells (B,D) were plated at $2x10^4$ cells per cm^2 on either cell culture plastic (A,B) or on collagen (C,D). Two days after plating, the soluble Jagged-1 transfectants formed multicellular chords on plastic (B) and collagen (D). Phase contrast x100.

Figure 3. Growth Kinetics of Soluble Jagged-1 and Insert-Less Vector NIH 3T3 Cell Transfectants. Cells were plated at a cell seed density of $1x10^4$ cells/cm^2 and counted daily in quadruplicate. Both vector and soluble Jagged-1 populations reach confluence at approximately four days after plating. Data are reported as the mean ± standard error of the mean.

Figure 4. Soluble Jagged-1 Tissue Mass Formation in Nude Mice. (A) Deep dermal view of a soluble jagged tissue mass 10 weeks after intradermal injection into the flank of a nude mouse. Note the prominent angiogenesis and the arborizing microvessels over the deep surface. (B) Hematoxylin and eosin stain of a paraffin section of the same tumor showing the prominent surface blood filled capillaries, penetrating vessels, and intra-tumor blood islands; magnification

is 100X. (C) Low magnification view (100X) of a frozen section of the same tumor showing immunohistochemical localization of CD31 (PECAM). Two cross sections of a microvessel are evident along with a high density of CD31 positivity which, upon higher (500X) magnification (D) is comprised of groups of single cells or angulated collection of CD31-positive cells.

Figure 5. Induction of Angiogenesis in the Chicken CAM Assay. Vector-transfected control NIH 3T3 cells (A), soluble Jagged-1 NIH 3T3 cell transfectants (B) and FGF-2 (C) were assessed for their ability to induce angiogenesis in the CAM assay as described in Materials and Methods. These photographic images were quantitated (D) by computer-assisted morphometry including the FGF-2 negative control.

Table 1: Most Frequently Observed SAGE Tags. Tags correspond to the 10 base pairs of DNA sequence data immediately following the NlaIII cleavage site. The count refers to the number of instances the tag appears in the SAGE database. Accession numbers are the GenBank designations referring to the mRNA identified in the description column.

Tags Predominant in the Soluble Jagged-1 NIH 3T3 Cell Transfectants.			
Tag	*Count*	*Accession*	*Description*
TGGATCAGTC	14	M62952	Mus musculus ribosomal protein L19
TAAAGAGGCC	9	U67770	Mus musculus ribosomal protein S26 (RPS26) mRNA
CCTGATCTTT	8	X06406	Mouse mRNA for translational controlled 40 kDa protein
TGTAACAGGA	8	X04648	Mouse mRNA for IgG1/IgG2b Fc receptor (FcR).
TCTGTGCACC	6	U93864	Mus musculus ribosomal protein S11 mRNA
CCAAATAAAA	6	U13687	Mus musculus DBA/2J lactate dehydrogenase-A
CTAATAAAAG	6	X54691	Mouse COX4 mRNA for cytochrome c oxidase subunit
GCCAAGGGTC	5	L08651	Mus musculus large ribosomal subunit protein mRNA
GTCTGCTGAT	5	X75313	M.musculus (C57BL/6) GB-like mRNA.
AAGGAAGAGA	4	X51438	Mouse mRNA for vimentin.
TGAAATAAAC	4	M33212	Mouse nucleolar protein N038 mRNA
CACCACCACA	4	X05021	Murine mRNA with homology to yeast L29 ribosomal prot.
CCTCAGCCTG	4	X52886	M.musculus mRNA for cathepsin D.
CTCTGACTTA	4	Y16256	Mus musculus mRNA for basigin
GTGGGCGTGT	4	M33330	Mouse insulinoma (rig) mRNA
TCCTTGGGGG	4	U60001	Mus musculus protein kinase C inhibitor (mPKCI) mRNA

Tags Predominant in the Control Vector NIH 3T3 Cell Transfectants.			
Tag	*Count*	*Accession*	*Description*
CGCCTGCTAG	3	X58251	Mouse COL1A2 mRNA for pro-alpha-2(I) collagen.
AAAAAAAAAA	2	AF0253	Mus musculus tssk-1 and tssk-2 kinase substrate mRNA
AAGCAGAAGG	2	M16465	Mouse calpactin I light chain (p11) mRNA complete
CAGGACTCCG	2	M26270	Mouse stearoyl-CoA desaturase (SCD2) mRNA
GAAGCAGGAC	2	D00472	Mouse mRNA for cofilin
GGATATGTGG	2	M20157	Mouse Egr-1 mRNA
GTTCTGATTG	2	U88588	Mus musculus cdr2 mRNA

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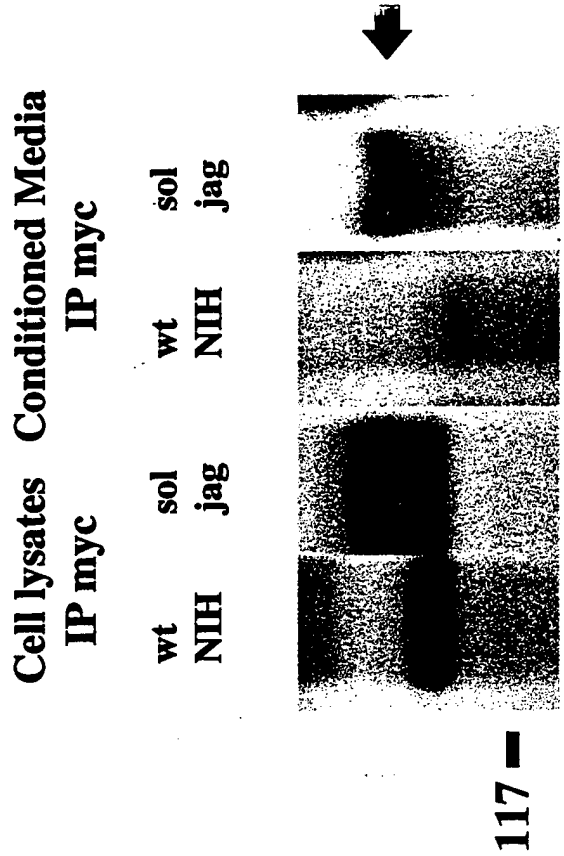
Figure 1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wt
NIH
sol
jag
Conditioned Media
IP myc
wt
NIH
sol
jag
117

Figure 2

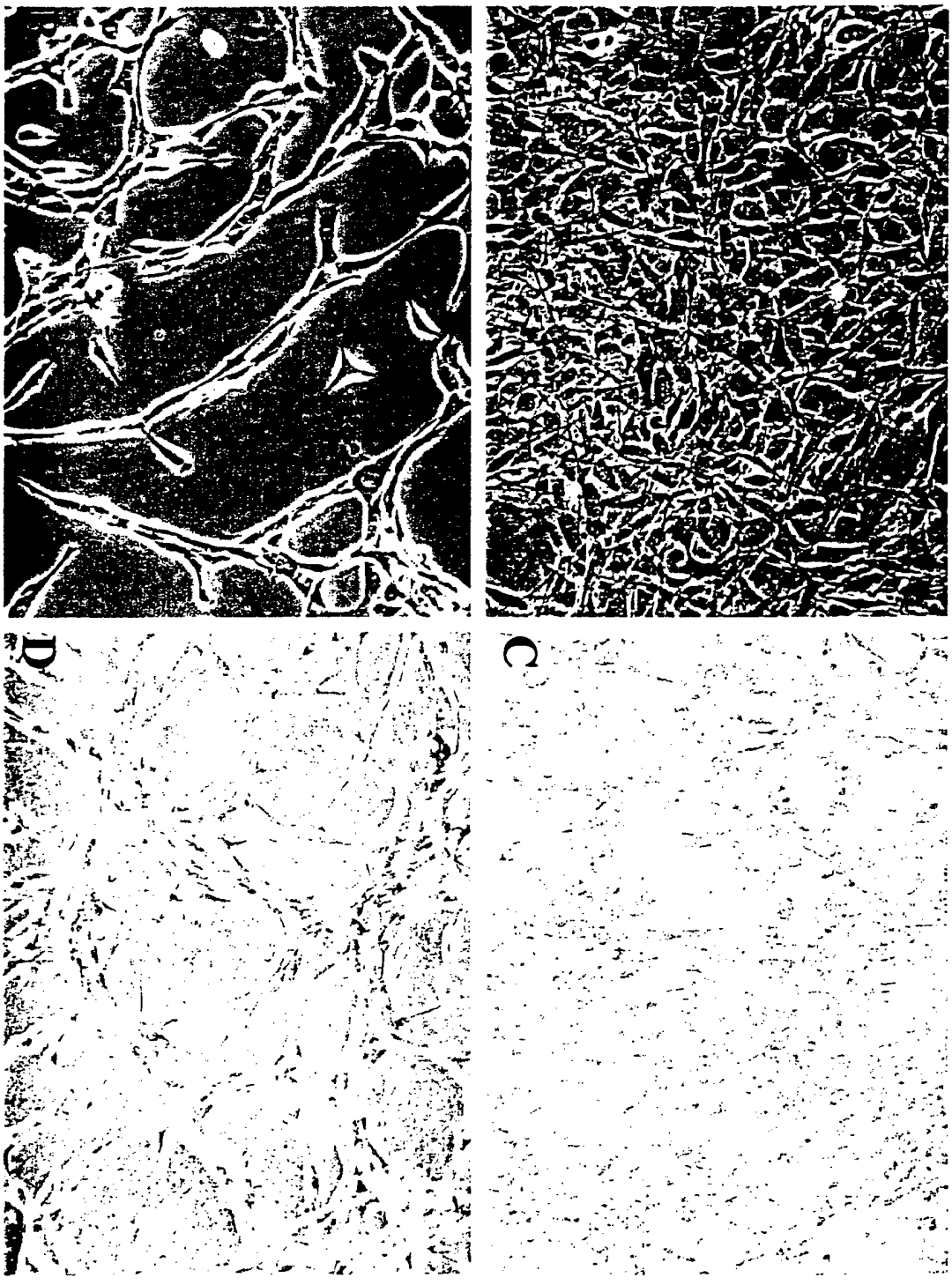
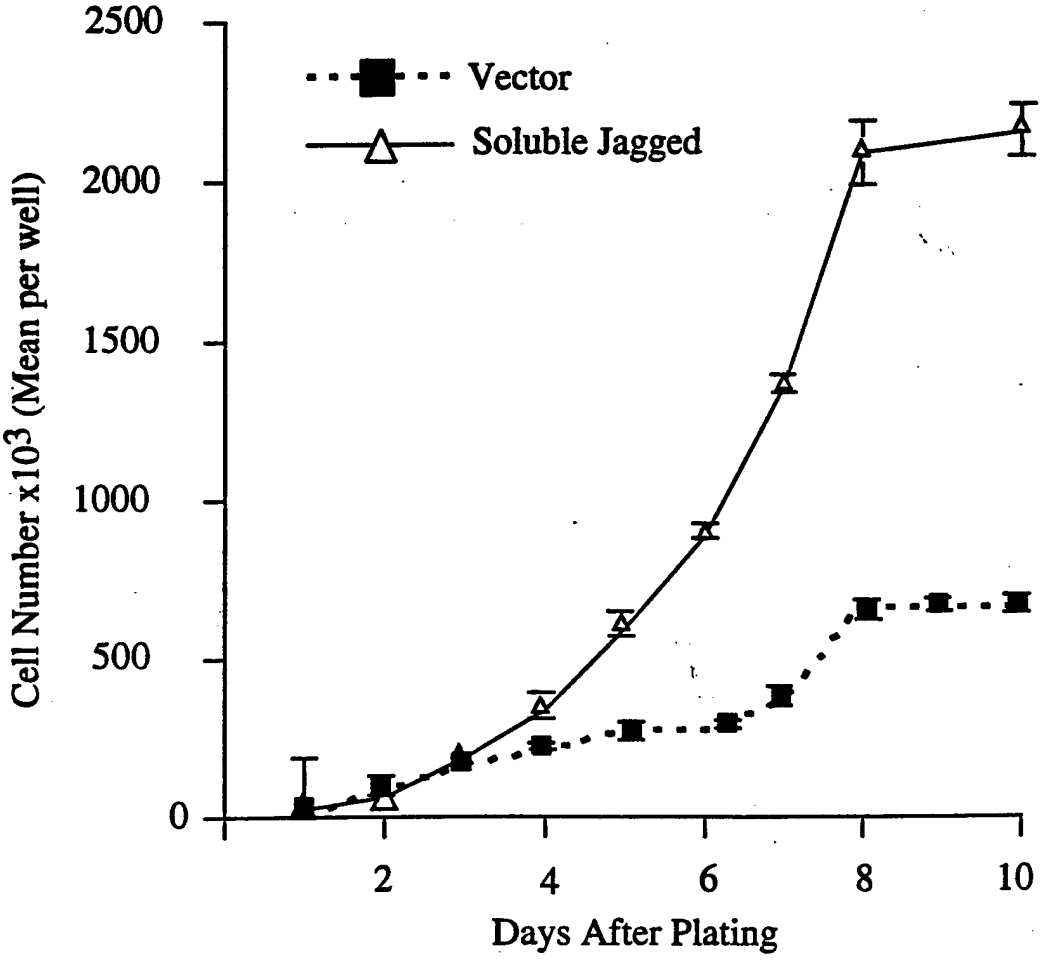


Figure 3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2000
1500
1000
500
0
Cell Number x10³ (Mean per well)
Vector
Soluble Jagged
2
4
6
8
10
Days After Plating

Figure 4

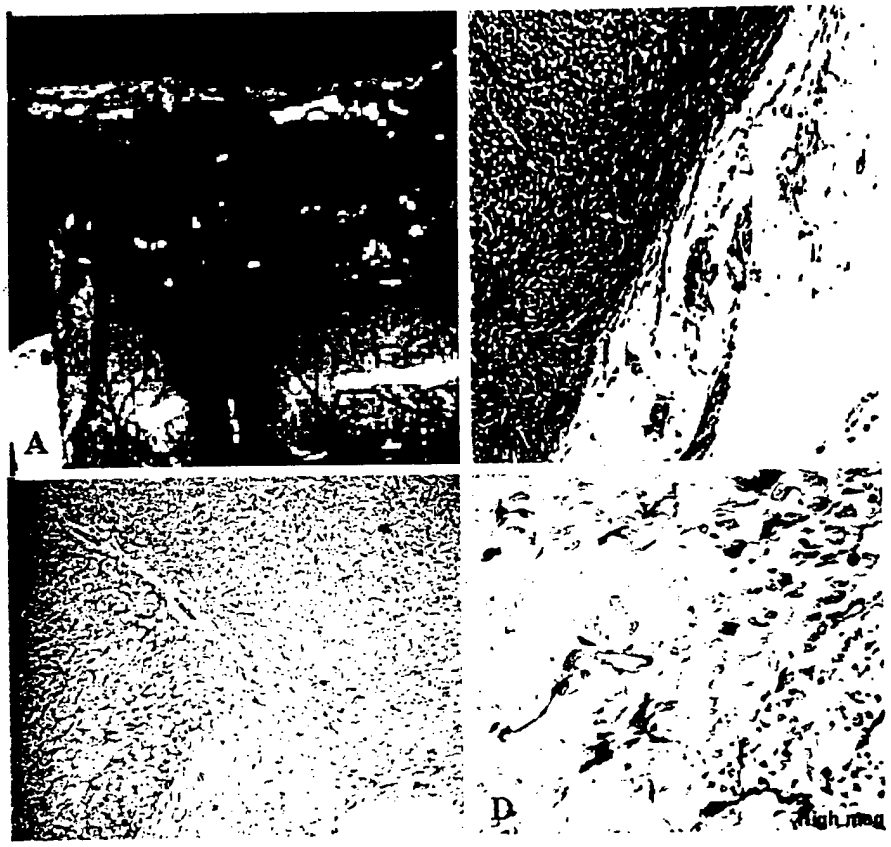
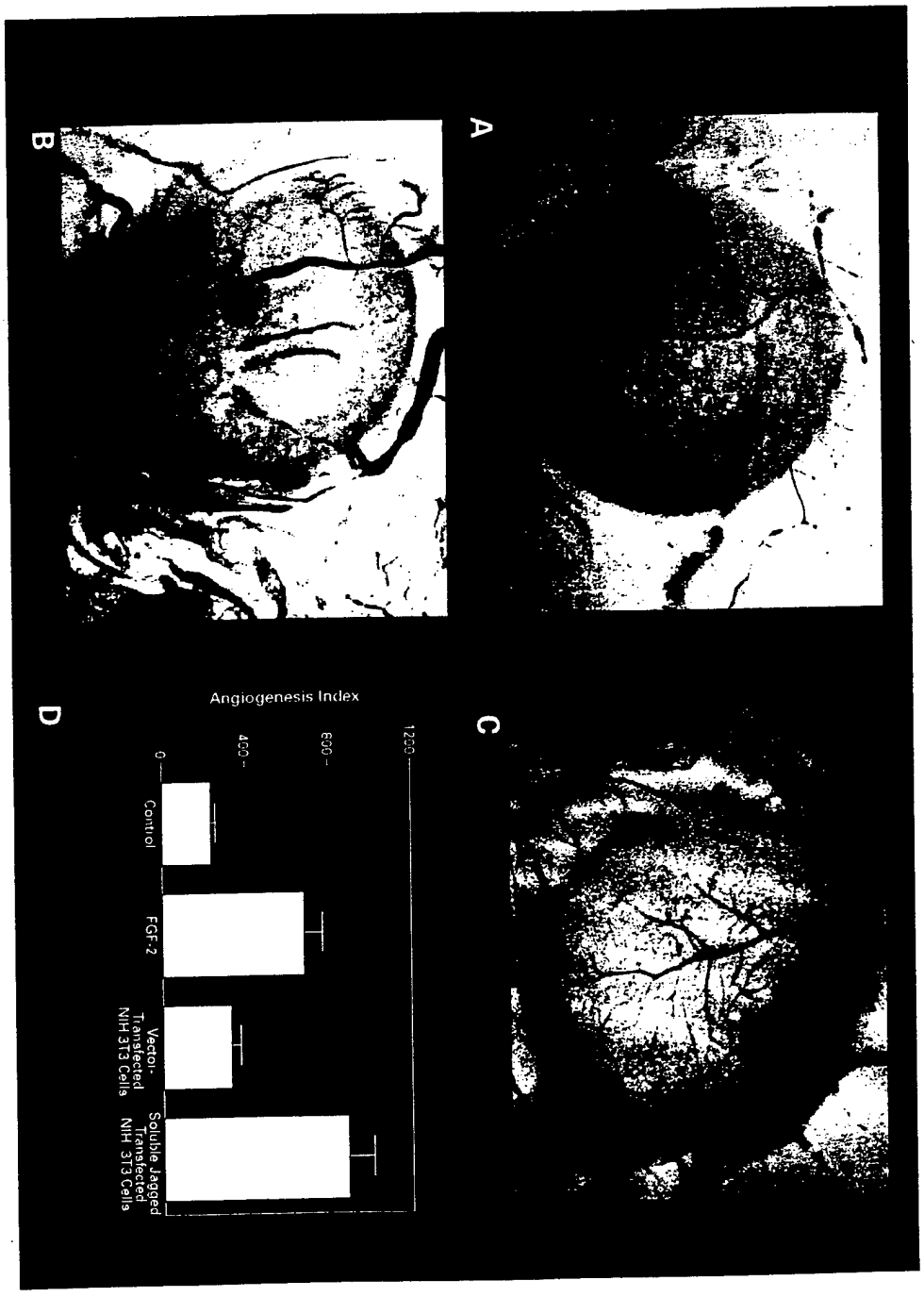


Figure 5


A
B
C
D
Angiogenesis Index
0
400
800
1200
Control
FGF-2
Vector-Transfected NIH 3T3 Cells
Soluble Jagged Transfected NIH 3T3 Cells